Fate of lysostaphin in milk from individual cows through pasteurization and cheesemaking[1]

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ABSTRACT

Transgenic cows secreting over 3 µg of lysostaphin/mL of milk are protected against mastitis caused by *Staphylococcus aureus*, but it is unknown if active lysostaphin persists through dairy processing procedures or affects the production of fermented dairy foods. The objective of this study was to determine the fate of lysostaphin as milk was pasteurized and then processed into cheese. Raw milk from transgenic cows was heat treated at 63°C for 30 min, 72°C for 15 s (high temperature, short time), or 140°C for 2 s (UHT). Portions of the high temperature, short-time milk were manufactured into semi-hard cheeses. Aliquots taken at each processing step were assayed to determine the quantity (ELISA) and activity (ability to inhibit *S. aureus* growth) of lysostaphin. Results indicated that most of the lysostaphin was present in the aqueous portion of the milk and was not affected by pasteurization, although UHT treatment reduced enzyme concentration by 60%. The quantity and activity of the lysostaphin decreased during cheesemaking. Based on the amount of lysostaphin present in the starting cheesemilk, 10 to 15% of the lysostaphin was recovered in the whey, 21 to 55% in the cheese curd at d 1, and 21 to 36% in cheese stored at 4°C for 90 d. Enough of the lysostaphin secreted into milk by transgenic cows survived typical dairy processing conditions to impart potential value as a bioprotective agent against staphylococci in dairy foods.

Key words: milk, lysostaphin, processing, transgenic cow

INTRODUCTION

Mastitis, bacterial infection of the mammary gland, has a negative impact on animal well-being as well as

diminishing both the productivity and milk quality of the individual cows. Reducing the incidence of mastitis, in addition to improving animal health, would be increased profitability by decreasing the 2 billion dollars that dairy farmers spend each year to address the consequences of mastitis (Rainard, 2005). Infections caused by *Staphylococcus aureus* are responsible for one-third of all the mastitis cases in the United States and are particularly difficult to treat when the infections are established deep in the mammary gland where infused antibiotics cannot penetrate (Sutra and Poutrel, 1994).

Lysostaphin (EC 3.4.24.75), a glycyl-glycine endopeptidase originally isolated from *Staphylococcus simulans*, specifically acts on the interpeptide pentaglycine bridges present in the cell wall of staphylococci, causing lysis and death of the organism (Browder et al., 1965). Lysostaphin activity is measured by its ability to lyses *S. aureus* cells and is influenced by enzyme concentration, pH, temperature, and ion and salt concentration (Schindler and Schuhardt, 1965). Because lysostaphin is specifically active against staphylococci, it may be useful in the control of these pathogens in many different applications. Lysostaphin may be used in presumptive tests to differentiate between staphylococcus and micrococci (Langlois et al., 1988). It is also effective in pharmaceutical applications including topical, subcutaneous, and intraperitoneal treatment of *S. aureus*-induced infections in rats and rabbits (Harrison and Cropp, 1967) and in controlling nasal colonization in mice (Kokai-Kun et al., 2003) and humans (Quickel et al., 1971). A few studies suggest incorporation of lysostaphin into foods to control *S. aureus* food poisoning, directly (Metcalf and Deibel, 1969) or through genetic engineering of lactic acid bacteria commonly used in food fermentations (Gaier et al., 1992; Cavadini et al., 1998).

Lysostaphin is partially effective in treating *S. aureus*-induced mastitis when infused directly into bovine udders (Oldham and Daley, 1991) but is impractical as an infusion product for a variety of reasons. Transgenic techniques can alter the ability of animals to produce specialty milks, such as enhancing resistance
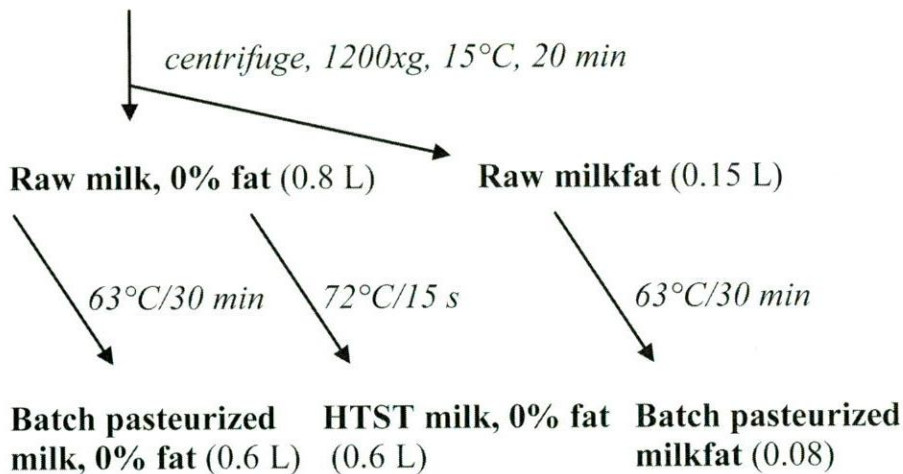
a) **Raw whole milk** (1.0 L)   [shipment 1: cow 101$_T$; shipment 2: cows 215$_T$ and 301$_C$]

centrifuge, 1200xg, 15°C, 20 min

Raw milk, 0% fat (0.8 L)   **Raw milkfat** (0.15 L)

63°C/30 min   *72°C/15 s*   *63°C/30 min*

Batch pasteurized   HTST milk, 0% fat   Batch pasteurized
milk, 0% fat (0.6 L)   (0.6 L)   **milkfat** (0.08)

b) **Raw whole milk** (50 L) [shipment 3: cows 312T, 310C; shipment 4: cows 101T, 215C]

Separator

Raw milk, 0% fat (30 L)   **Raw cream, 40% fat** (1 L)

130°c/2 s

UHT milk, 0% fat (1 L)

130°c/2 s   **UHT milk, 3% fat** (1 L)

Raw milk, 3% fat   *63°C/30 min*
(23 L for shipment 3,
45 L for shipment 4)   **Batch pasteurized milk, 3% fat** (0.5 L)

72°C/15 s   *72°C/15 s*

HTST milk, 0% fat (22 L for shipment 3)

HTST milk, 3% fat
(22 L for shipment 3,   Used to make cheese
45 L for shipment 4)
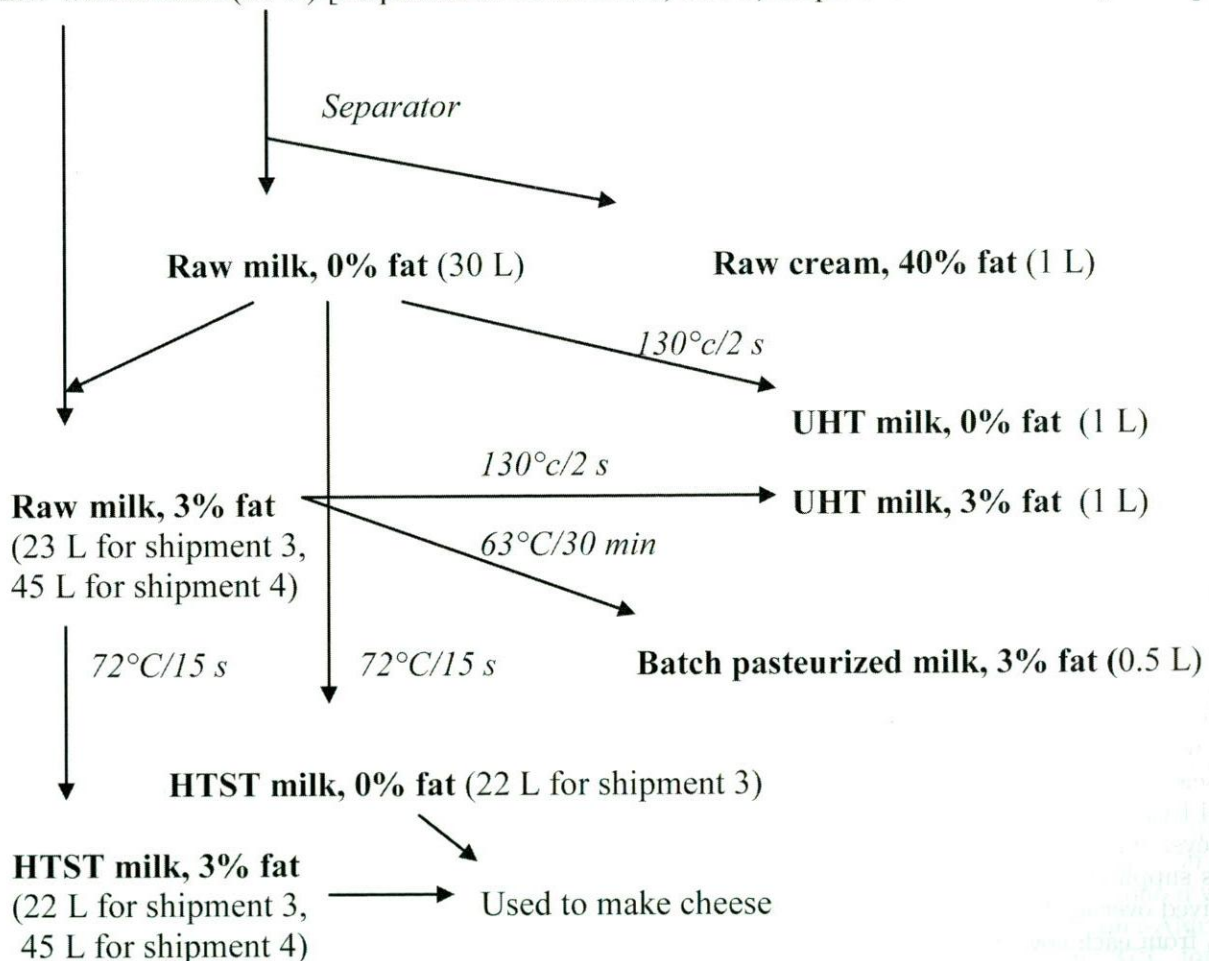
Figure 1. Flowchart of the processing of the milk from the 4 shipments: a) 1 L for heat treatment and b) 50 L for UHT heat treatment and cheesemilk.

to mastitis-causing bacteria. Transgenic mice carrying a β-LG-lysostaphin fusion gene secrete lysostaphin at concentrations of 0.06 to 1.3 mg/mL of milk. Their resistance to *S. aureus*-induced mastitis improves with increasing lysostaphin concentrations (Kerr et al., 2001). Transgenic cows producing as little as 3 μg of lysostaphin/mL of milk appear to be completely protected against repeated intramammary challenges with *S. aureus* (Wall et al., 2005). Secretion of recombinant lysostaphin in the milk is stable throughout lactation, and milk compositions and yields were similar between transgenic and nontransgenic herd mates.

Information about incorporating lysostaphin into dairy products is limited. In one study, bacterial lysostaphin added to *S. aureus*-spiked pasteurized milk initially reduced the *S. aureus* population in the cheese, but the population eventually thrived (Metcalf and Deibel, 1969). It was concluded that the decreasing pH found during cheese manufacture influenced the effectiveness of the lysostaphin. Addition of lysostaphin to bovine milk indicated that bacterial lysostaphin did not degrade milk proteins or hinder the production of yogurt made with commercial starter cultures (Kerr and Wellnitz, 2003). It was not known if the elevated temperatures typically used in dairy processing or the changing environment (decreasing pH or exposure to chymosin) found during cheesemaking and cheese aging would alter the biological activity of lysostaphin secreted by transgenic cattle or if the presence of the lysostaphin would affect cheesemaking properties. Therefore, the objectives of this study were to determine 1) the impact of heat processing on the lysostaphin activity in milk from transgenic cows, 2) the distribution of lysostaphin at different cheesemaking steps, 3) the persistence of active lysostaphin in fresh whey and in cheese stored at 4°C for up to 90 d, and 4) the effect of lysostaphin on cheesemaking properties.

MATERIALS AND METHODS

Milk

Fresh milk was obtained from individual cows (3 transgenic cows, 101_T, 215_T, and 312_T; and 3 nontransgenic control cows, 207_C, 301_C, and 310_C; cows were 5 to 8 wk postpartum) from the dairy herd at the Animal Bioscience and Biotechnology Lab, Beltsville Agricultural Research Center (Beltsville, MD). Five cows were Jerseys, and one cow, 215_T, was a Jersey mix. Different cows supplied the samples for the 4 shipments of milk received over an 18-mo period (Figure 1). Milk samples (1 L from each cow) in the first 2 shipments were used in the pasteurization study and included milk from 101_T (first shipment), and 215_T and 301_C (second shipment).

Milk samples (50 L from each cow) in the last 2 shipments were also used in the pasteurization study and to make cheese and included milk from 310_C and 312_T (third shipment) and 207_C and 101_T (fourth shipment). Milk samples were refrigerated (4°C) before being packed in ice and transported to the Dairy Processing and Products Research Unit facility (Wyndmoor, PA). Processing began immediately upon arrival.

Processing of Milk and the Manufacture of Cheese

Milk samples from individual cows were processed separately within 24 h of harvest (Figure 1). Aliquots were obtained at each processing step for compositional analyses and lysostaphin assays. Raw whole milks from the first and second shipments (Figure 1a) were centrifuged (RB5, Dupont, Wilmington, DE) at 1,200 × *g* for 20 min at 15°C and the milkfat removed. The skim milk portion (0% fat) was batch-pasteurized in a loosely capped bottle in a water bath set at 63°C for 30 min or was HTST-pasteurized at 72°C for 15 s using a HTST/UHT Heat Exchanger Processing unit with a recirculating chiller (FT 74 P and FT 63, Armfield, Jackson, NJ). Milk fat portion was batch-pasteurized.

Over one-half of the raw whole milks from the third and fourth shipments (Figure 1b) were warmed to 35°C and separated into cream or skim milk portions using a disc bowl centrifuge (Counter top model, Armfield). The whole and skim milks were blended to obtain a standardized 3% fat milk. Small portions of the 0 and 3% fat milks were batch-pasteurized or UHT-processed using the Armfield HTST/UHT unit, whereas the bulk of the milks were pasteurized using the HTST method and stored overnight at 4°C.

Milk from the same cow, adjusted to 0 or 3% fat before HTST pasteurization, was processed into semi-hard cheese using a modified procedure based on the Cheddar cheese as described by Kosikowski and Mistry (1997). A mesophilic starter culture (R-604; 40 mL of 1 frozen culture pellets:10 milk; Chr. Hansen, Milwaukee, WI) and microbial chymosin (0.7 mL, diluted 1:40; Chr. Hansen) were used in cheesemaking. Two side-by-side vats (22-kg capacity each) were used each day. Modifications included allowing milk to coagulate for 35 to 40 min before it was cut with 20-mm knives. Stacked curd was turned 3 times every 15 min, then hand cut into 12- to 15-mm pieces, and packed into plastic rectangular molds (10 × 10 × 25 cm) for overnight pressing at 138 kPa at 22°C. Weights of cheese milk, whey (from vat and from the press), and curd (after pressing) were determined. Cheeses were removed from the mold, vacuum packaged, placed in 4°C storage, and sampled every 30 d for up to 90 d.

The third shipment consisted of milk from a nontransgenic control cow (310_C) and a transgenic cow (312_T), estimated to be secreting 2 μg of lysostaphin/mL of milk. Each cheese vat was filled with 9.07 kg of HTST-pasteurized milk, and 4 lots of cheese were manufactured in 2 d included 2 control cheeses and 2 cheeses containing lysostaphin. The fourth shipment consisted of milk from a different nontransgenic control cow (207_C) and a transgenic cow (101_T) estimated to be secreting 8 μg of lysostaphin/mL. For this shipment, the milk was standardized to 3% fat, and 20 kg was used to make cheese. Concentrations of starter cultures and rennet were increased proportionally. The 2 cheeses manufactured on the same day included one nontransgenic control lot and one lot containing lysostaphin.

Determination of Lysostaphin Content and Activity

The ELISA was used to quantify the mass of recombinant lysostaphin in the samples as described by Kerr et al. (2001). The spot-on-lawn (**SOL**) method (Henderson et al., 1992) was used to detect the lysostaphin activity in stock solutions, milk samples, processing fractions, and cheese homogenates. Cheese samples were dispersed in distilled H_2O (1 cheese: 9 H_2O, wt/wt) and sonicated at 4°C (12 to 15 1-min bursts) to obtain suitable homogenates. After 2-fold serial dilution, 5 μL of samples were spotted on tryptic soy agar films (Difco Laboratories, Detroit, MI) inoculated by spreading with *S. aureus* (10^6 cfu/mL). Plates were incubated for 16 h at 37°C and checked for the presence of clear inhibition zones indicating lysostaphin activity. An arbitrary lysostaphin activity unit was defined as the greatest dilution of each sample yielding a visible zone of inhibition in the SOL assay. Total activity, calculated by the reciprocal of the highest dilution value, was given in arbitrary lysostaphin activity units in the SOL assay per milliliter or gram.

The MIC assay also was used to detect lysostaphin activity in the cheese study. Milk samples were used directly, whereas cheese samples were dispersed in reconstituted nonfat dry milk (Difco Laboratories) in a 5:1 (wt/vol) ratio and homogenized for 60 s at 30,000 rpm (PT3000; Brinkman Instruments, Westbury, NY). Falcon 96-well plates were prepared with 100 μL of tryptic soy broth (Difco Laboratories), and samples were 2-fold serial-diluted in the plate. A 50-μL aliquot of log phase *S. aureus* grown in brain heart infusion (Difco Laboratories) and diluted 1:1,000 was added to each well and plates incubated at 37°C for 18 h. An aliquot of bromocresol purple (25 μL of 0.25% in PBS; Becton Dickinson Bioscience, San Jose, CA) was added to each well; a purple color developed at pH 7.2, which disappeared as the pH decreased (no color at pH 4). An arbitrary lysostaphin activity unit was defined as the greatest dilution of each sample yielding a purple color in the MIC assay. Total activity was calculated by the reciprocal of the greatest dilution value and given in arbitrary lysostaphin activity units in the MIC assay per milliliter or gram.

Determination of Milk and Cheese Composition

Each fraction collected in this study was assayed for composition. Moisture was determined in triplicate using the forced-draft oven method (method 948.12; AOAC, 2000). Fat was measured in duplicate using a modified Babcock method (Kosikowski and Mistry, 1997). Total nitrogen was obtained in duplicate using a FP-2000 nitrogen analyzer (Leco Corp., St. Joseph, MI), and total protein was calculated by multiplying the percentage of nitrogen value by 6.38 (method 920.123; AOAC, 2000). Lactose was determined in duplicate directly from liquid samples or from warm water filtrates from each solid sample using a Lactose analyzer (Application Note 320, model YSL 2700 Select, YSI US, Yellow Springs, OH). Ash was determined in duplicate using a gravimetric method (method 935.42; AOAC, 2000). Alkaline phosphatase activity was measured in duplicate using a Charm Pas Lite test (Charm Sciences Inc., Lawrence, MA) to verify inactivation of the enzyme in pasteurized samples (Rocco, 1990). The pH of each cheese was measured 6 times (Orion 611, Research Inc., Cambridge, MA).

Protein Profiles and Physical Properties

Protein profiles of all fractions were examined using SDS-PAGE. Milk samples were diluted directly into Tris-SDS buffer while proteins were extracted from the solid portions (curd and cheeses) according to the method described by Tunick et al. (1995). Proteins were separated on 20% homogeneous ultrathin gels using a PhastSystem (GE Healthcare, Piscataway, NJ) and stained with Coomassie blue. Gels were scanned and protein distribution calculated using a 375A Personal Densitometer SI equipped with Image Quant software, Version 4.2 (Molecular Dynamics, Sunnyvale, CA). Major bands were identified as α_{S1}-CN, α_{S2}-CN, β-CN, κ-CN, α-LA, and β-LG.

The rennet interaction was tested in control experiments where bacterial lysostaphin (12.5, 25, and 50 μg/mL; Sigma-Aldrich, St. Louis, MO) was incubated with chymosin (0, 7.8, 15.5, and 31 μg/mL; Sigma-Aldrich) in a 0.01 *M* phosphate buffer, pH 6.0 at 37°C for 90 min. Activity of the lysostaphin was tested using the SOL method.

The rates of coagulation for the milks prepared from the third and fourth shipments were determined in duplicate using a dynamic analyzer (AR2000, TA Instruments, New Castle, DE). A 20-mL aliquot of HTST pasteurized milk containing 0 or 3% fat (stored at −35°C before testing, warmed to room temperature; pH 6.3 to 6.4) was placed in a concentric cylinder (Couette) maintained at 32°C and allowed to warm for 5 min. A 100-µL aliquot of microbial chymosin (diluted 1:40; Chr. Hansen) was mixed into the milk before lowering the inner cylinder. A time sweep at 1.0 Hz (6.284 rd/s) was started 65 s after the addition of the enzyme and ended when the elastic modulus reached 20 Pa. The coagulation time was recorded at the point where the elastic modulus became greater than the viscous modulus.

The melt characteristics of the cheeses were determined using the Schreiber melt test as described by Kosikowski and Mistry (1997). Three disks (12 mm in diameter, 4 mm in height) were heated in a 232°C oven for 5 min. After cooling, the disk area was measured (n = 6) using a concentric ring grid and the increase in surface area determined; scores of 1.0 indicated the sample did not melt and flow. The whiteness (L*) value of the disks were measured (n = 4) before and after the melt test using a Hunter Lab (ColorQuest XE Colorimetric Spectrophotometer; Hunter Associates Laboratory, Reston, VA).

Rheological properties were measured on cheeses after 10 d of storage at 4°C as described by Van Hekken et al. (2007). Torsion analysis (4 repetitions) was conducted using a torsion gelometer (Gel Consultants Inc., Raleigh, NC) operating at 2.5 rpm. Gelometer software collected shear stress and shear strain at point of fracture and calculated the shear rigidity (ratio of stress to strain) at the point of fracture. Texture profile analysis (4 repetitions) was conducted using a universal testing machine (Sintech, Model SM-25–155, Material Testing Products Systems Corp., Eden Prairie, MN); double 75% compression was conducted at a crosshead speed of 100 mm/min. Hardness, cohesiveness, and springiness were calculated by the instrument's software from the generated force-distance curves. Small amplitude oscillatory shear analysis (3 repetitions) was conducted using a dynamic analyzer (AR2000 rheometer, TA Instruments, New Castle, DE). Frequency sweeps (0.1 to 100 rd/s) were conducted at 0.8% strain.

Statistical Evaluation

Statistical analyses of compositional and ELISA data were performed by ANOVA with mean separation using the Bonferroni LSD technique, $P < 0.05$ (SAS Institute, 2001). Based on the data set, the appropriate major

effects tested included lysostaphin presence, individual cow, fat content of milk, fraction, age of cheese, and different interactions.

RESULTS AND DISCUSSION

Lysostaphin Activity Against Staphylococci

The standard assay to determine lysostaphin activity is based on the reduction of turbidity of *S. aureus* suspended in a phosphate buffer. Because of the opaque nature of milk and the particulate matter in cheese homogenates, alternate assays were used to detect lysostaphin activity. Both the SOL and MIC assays successfully illustrated the reduction in lysostaphin activity as the samples were diluted. In the SOL assay, as the amount of the lysostaphin decreased with dilution, the inhibition zones decreased in size and then went from clear to cloudy as *S. aureus* began to grow within the area (Figures 2 and 3). For the more concentrated samples, the inhibition zones were a clear ring around the opaque milk droplet. The MIC assay was developed to work with opaque and particulate samples, especially the homogenized cheese samples. The pH indicator dye remained purple until the lysostaphin concentration decreased to an ineffective level that allowed the *S. aureus* to grow and produce acids. As the pH within the well decreased, the purple color faded and gave a sharper dilution endpoint to detect lysostaphin activity (Figure 4). Lysostaphin activity was estimated in arbitrary lysostaphin units (**ALU**) from the highest dilution value that still showed some bacteriolytic activity toward *S. aureus*. The activity varied between the 2 assays with the SOL activities typically being 20-fold higher.
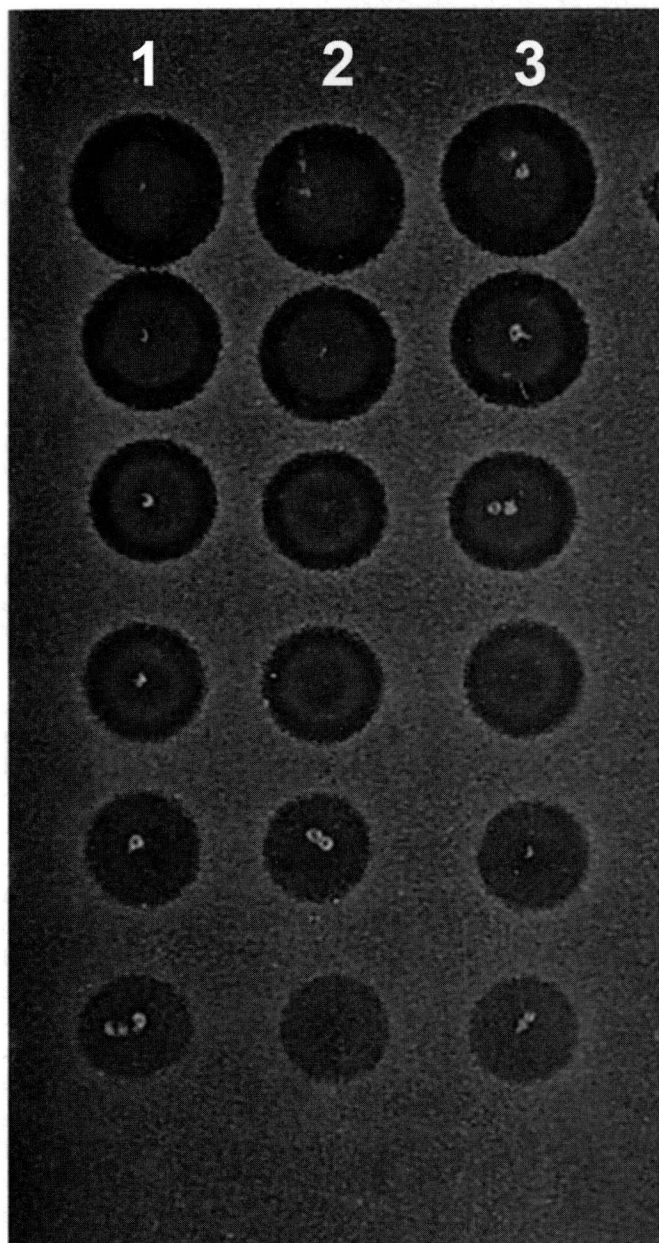
Heat Treatment of Milk

Pasteurization of milk had minimal affect on lysostaphin activity in whole, 3% fat, and skim milk as measured by its ability to lysis *S. aureus* cell walls. No differences were observed in lysostaphin activity in the SOL assay among raw, batch-pasteurized, and HTST-pasteurized skim milk from cow 101_T where activity remained detectable following a 1:32 dilution (Figure 2, columns 1, 2, and 3). An expanded dilution of heat treated milk from cow 101_T (Figure 3, columns 1, 2, 4, and 5), showed activities of the raw and pasteurized milk of 102,400 ALU/mL. A similar trend was noted among the raw and pasteurized fractions from cow 312_T as seen in the MIC assay (Figure 4, rows 5 and 6) with activities estimated at 10,240 ALU/mL. The quantities of lysostaphin measured in milks of similar fat content were similar ($P > 0.05$) for raw milk, and batch- and HTST-pasteurized milk but decreased significantly (P

< 0.05) after UHT processing (Table 1). Over 60% of the enzyme was destroyed (no longer identified by ELISA) and MIC activity was reduced to 35 to 40% of the raw milk sample.

Pasteurization is a standard practice in the American dairy industry. The Code of Federal Registration (2003) defines pasteurization as heat treating every particle in the milk/dairy product for a specific temperature/time, such as 63°C for 30 min or 71.7°C for 15 s. The primary

Figure 2. Spot-on-lawn assay showed similar activity of lysostaphin in 1) raw skim milk, and skim milk pasteurized at 2) 63°C for 30 min, and 3) 72°C for 15 s; 2-fold serial dilution of samples (top to bottom).

target of the heat treatment is to destroy pathogens and reduce spoilage microorganisms. The HTST pasteurization temperatures can range from 72 to 80°C for at least 15 s (McKellar et al., 1996) and are known to inactivate some indigenous milk enzymes such as alkaline phosphatase (Fox and Cogan, 2004), lactoperoxidase, and γ-glutamyl transpeptidase (McKellar et al., 1996). Other indigenous milk enzymes are known to tolerate HTST pasteurization temperatures, including plasmin, acid phosphatase, and xanthine oxidase (Fox and Cogan, 2004). Although it is estimated that milk contains around 60 indigenous enzymes, only 20 have been identified (Fox and Cogan, 2004). Even fewer have shown the potential to impact cheese quality; including plasmin, lipoprotein lipase, proteinases, acid and alkaline phosphatase, xanthine oxidase, sulphydryl oxidase, lactoperoxidase, and γ-glutamyl transpeptidase. The greater UHT temperatures will also denature whey proteins, which alter protein solubility and functional properties (Douglas et al., 1981).

Pasteurization of the milk at 63°C for 30 min or 72°C for 15 s did not alter lysostaphin levels or activities when examined by SOL or MIC assays. This does not agree with an earlier study (Schindler and Schuhardt, 1965) that reported an approximately 50% decrease in activity of bacterial lysostaphin (isolated and purified in their laboratory) in buffered saline following a 30-min exposure at 60°C and complete inactivation of the enzyme following a 15-min exposure at 75°C. Schindler and Schuhardt (1965) did note that a buffered solution protected the activity of the enzyme somewhat. Milk is a well-known buffering solution and is more complex than the saline solution used in the Schindler and Schuhardt study. Our findings did agree with a more recent study (Donovan et al., 2006) in which the activity of commercial bacterial lysostaphin in water or a milk serum (milk ultracentrifuged to remove lipid and casein micelles) was not altered after heating at 63°C for 30 min (batch pasteurization); the study did not test higher temperatures. Of interest, recombinant lysostaphin (similar to the lysostaphin secreted by our cows) fused to B 30 phage endolysin was active in water and in the milk serum but was completely inactivated by batch pasteurization. It has been shown that lysostaphin from different sources (bacterial, mouse, and cow) have different activities (Kerr et al., 2001; Wall et al., 2005), and it may be that heat stabilities are also different. The components in milk could also be important factors in enhancing the enzyme's ability to withstand heat stress. Our study showed similar heat inactivation of lysostaphin in 0 and 3% fat milks; therefore, the lipid fraction may not factor into higher heat tolerance. The study by Donovan et al. (2006) indicated that the buffering power of the milk serum
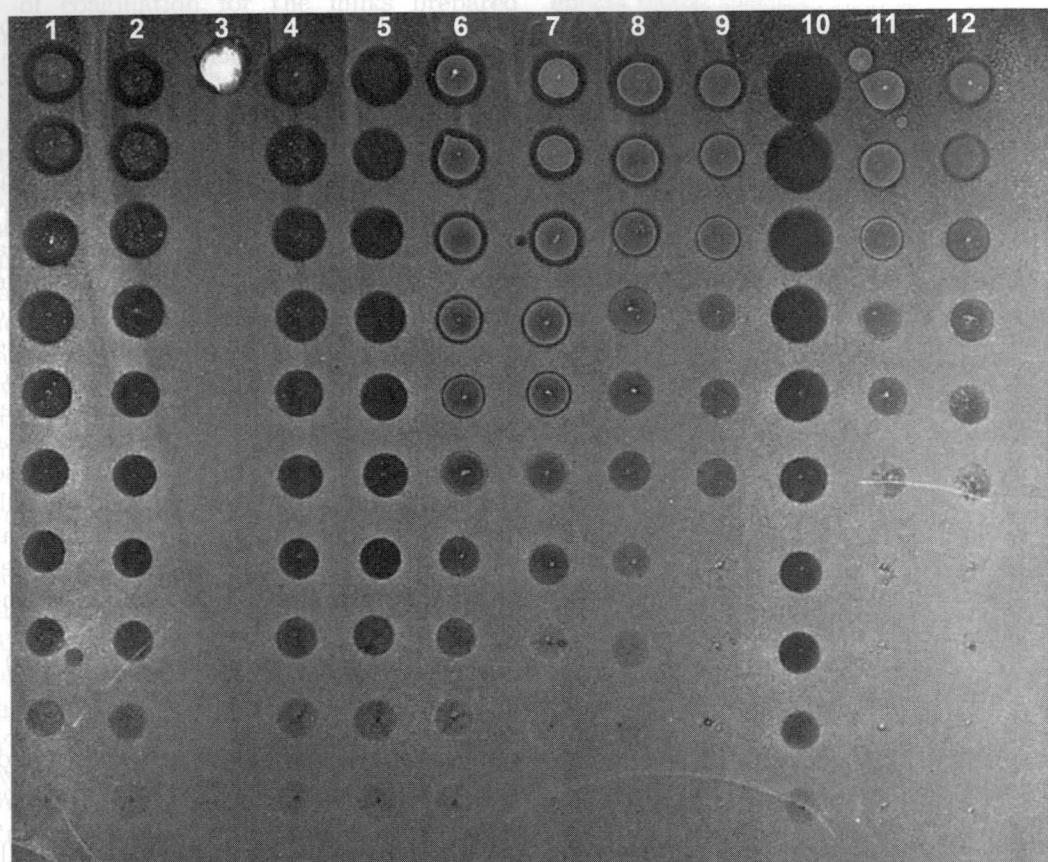
Figure 3. Spot-on-lawn assay showed the changes in recombinant lysostaphin acitivity in different fractions collected durning cheesemaking; 2-fold serial dilution (top to bottom). Fractions: 1) raw milk, 2) raw skim milk, 3) raw cream, 4) raw milk standardized to 3% fat, 5) pasteurized milk standardized to 3% fat, 6) ripened cheese milk, 30 min after the addition of the starter culture, 7) milk gel, 30 min after the addition of chymosin, 8) whey drained from the cheese vat, 9) fresh curd after draining whey, 10) whey from overnight pressing, 11) cheese curd after overnight pressing, and 12) cheese after 30 d of storage at 4°C; solid samples were homogenized 1:10 before serial dilution.

and the presence of whey proteins had little effect on the stability of lysostaphin activity at 63°C. That suggests that the casein micelles in milk may afford some protection to the lysostaphin when exposed to the higher temperatures. More research is needed to clearly understand the effect of heat on the activity and stability of bovine lysostaphin.

Modification of the fat content in milk is typical in dairy processing. Depending on the breed, herd averages can vary from 1.8 to 6% milk fat, with the Jersey breed averaging 4.6% (Heinrichs et al., 1997). Although pooling of milk on the farm and then at the processing plant results in more uniform fat content, the practice of standardizing milk fat content to 0 (skim), 1, 2 or 3.25% milk fat is widely used in the fluid milk industry. As expected, milk composition showed cow-to-cow variation, but the overall composition of the raw milk from the transgenic and nontransgenic cows were not different ($P < 0.05$; Table 2). Fat contents were the most variable among the cows and ranged from 3.0

to 5.4%, but were typical of the variation observed in milking herds. Cow 215_T, which was not a purebred Jersey, had the least fat. The milk from the transgenic cow, 101_T, had the highest milkfat and protein concentrations of all the cows in the study as well as the greatest lysostaphin concentration of the transgenic cows. In an earlier study, this cow had been proven resistant to challenges of *S. aureus* infused into the mammary gland (Wall et al., 2005). The other 2 transgenic cows in the study were either at or below the 3 μg/mL level of lysostaphin determined to protect the cows from mastitis caused by *S. aureus*. The cows in our study were clones specifically engineered to secrete lysostaphin in the milk. Other studies have shown that the composition of milk produced by cloned animals were not statistically different from noncloned animals (Norman et al., 2004; Tian et al., 2005).

Lysostaphin concentration and activity was not significantly different between the 0 and 3% fat milks from cow 312_T (Table 1). For cow 101_T, the raw fat fraction
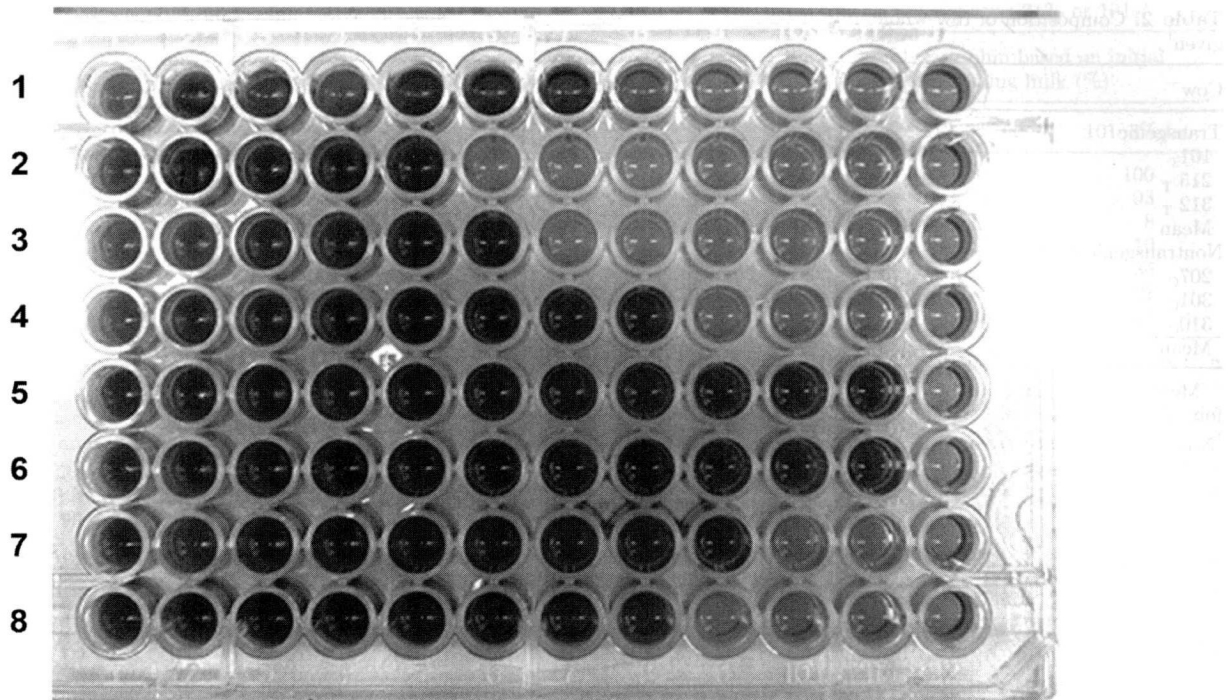
Figure 4. Minimum inhibitory concentration assay showed lysostaphin activity in milk and cheese made with 3% milk from transgenic cows; 2-fold serial dilution (left to right). Milk from the transgenic cow secreting 8.3 µg of lysostaphin/mL of milk [row 5) raw whole milk, and 6) pasteurized 3% fat] was made into cheese and aged at 4°C for 1) 0-d, 2) 30-d, 3) 60-d, and 4) 90-d. Milk from the transgenic cow secreting 1.7 µg of lysostaphin/mL milk [row 7) raw whole milk, and 8) raw 3% fat milk] is shown for comparison.

obtained from centrifugation (milk from the first shipment) did not have any detectable antibacterial activity (Figure 3, column 3). The raw cream fraction from cow 101_T (milk from the fourth shipment), which contained 40% fat, had only trace amounts of activity. This was attributed to the lysostaphin present in the aqueous portion of the cream and supports the Metcalf and Deibel (1969) conclusions that the bulk of the lysostaphin remained in the aqueous portion of the milk.

Cheese Manufacture

Overall, there were a few differences in processing the milks into cheese; most were batch-to-batch differences,

Table 1. Concentration and activity of lysostaphin in different milk samples from transgenic cow 312_T before and after different heat treatments; batch pasteurization conducted at 63°C for 30 min, HTST pasteurization conducted at 72°C for 15 s, and UHT sterilization conducted at 140°C for 2 s[1]

Milk sample (fat content/treatment)	Concentration of lysostaphin[2] (µg/mL)	Lysostaphin activity[3] (ALU/mL milk)
4.6%/raw	1.70[a] (0.02)	1,280
3%/raw	1.19[ab] (0.06)	640
3%/batch	1.20[ab] (0.06)	640
3%/HTST	1.11[ab] (0.08)	640
3%/UHT	0.41[c] (0.09)	160
0%/raw	0.92[ab] (0.08)	640
0%/batch	ND[4]	640
0%/HTST	0.91[ab] (0.09)	640
0%/UHT	0.36[c] (0.08)	160

[a-c]Means in the same column with different letters are different ($P < 0.05$).

[1]Numbers in parentheses are SEM.

[2]Lysostaphin concentration determined using ELISA assay.

[3]Lysostaphin activity calculated from MIC assay and given in arbitrary lysostaphin units (ALU).

[4]ND = not determined.

Table 2. Composition of raw whole milk from nontransgenic herd mates and transgenic cows; individual cow averages and overall means are given[1]

Cow	Moisture (%)	Fat (%)	Protein (%)	Lactose (%)	Lysostaphin (μg/mL)
Transgenic					
101_T	85.8^b (1.0)	5.2^a (0.3)	3.6 (0.7)	4.8^a (0.3)	8.3 (0.3)
215_T	88.0^a (0.1)	3.2^d (0.1)	3.5 (0.1)	4.7^a (0.1)	3.8 (0.1)
312_T	86.8^{ab} (0.4)	4.6^b (0.2)	3.4 (0.1)	4.6^a (0.1)	1.7 (0.1)
Mean	86.9 (0.6)	4.3 (0.5)	3.5 (0.2)	4.7 (0.1)	
Nontransgenic control					
207_C	86.8^{ab} (0.1)	4.6^b (0.1)	3.6 (0.1)	4.4^b (0.2)	0.0
301_C	87.3^{ab} (0.5)	3.6^c (0.1)	3.7 (0.1)	4.8^a (0.1)	0.0
310_C	86.4^b (0.1)	4.5^b (0.1)	3.8 (0.1)	4.7^a (0.1)	0.0
Mean	86.8 (0.3)	4.2 (0.3)	3.7 (0.2)	4.6 (0.1)	

[a-d]Means in the same column with different letters are different ($P < 0.05$); the lack of letters in the same column indicates no differences were found.

[1]Numbers in parentheses are SEM.

although some were cow-to-cow differences. No trends were observed that indicated that differences were because of the presence or lack of lysostaphin in the milk (Table 3). Although all milks were at pH 6.6 at the start of cheesemaking, milks from the third shipment (cows 312_T and 310_C) required 60 to 65 min of incubation with the starter culture to decrease titratable acidity by 0.1 unit before rennet was added. Milk from cow 310_C responded slower in acid production initially but recovered by the time the whey was drained. Milk from the fourth shipment (cows 101_T and 207_C) required only 30 min for the pH to reduce by 0.1 units and titratable acidity by 0.2 units; volumes in cheese vats were twice the amount used for the third shipment milks (20 vs. 9.07 kg, respectively). The faster pH development for the fourth shipment milks continued as the pH at the time of whey draining was also lower than the third shipment milks (5.8 to 5.9 compared with 6.3 to 6.4,

respectively). Because the cheeses made within 48 h of each other had similar acid development, the differences noted between cheeses made from third or fourth shipment milks were unlikely to be due to an adverse affect of lysostaphin present in the milk. Lysostaphin activity is very specific toward staphylococci and was not expected to have any activity toward the common dairy starter cultures. Kerr and Wellnitz (2003) mentioned that addition of up to 100 μg of bacterial lysostaphin/mL of milk had no effect on yogurt production. Batches of cheese made side-by-side on the same day had similar conditions, but the yield varied by cow. The nonfat cheeses typically lost 35 to 40% of curd weight during milling and overnight pressing and had the least yields (under 8%). The 3% fat milk cheeses lost between 23 to 30% of curd weight during milling and overnight pressing, and ranged from 8 to 11% yield; 10% yield is typical in cheese production. Between the paired milks

Table 3. Cheesemaking details for milk from transgenic cows (312_T and 101_T) and control cows (310_C and 207_C)

	0% cheese milk		3% milk fat			
Item	312_T	310_C	312_T	310_C	101_T	207_C
Starting weight of milk (kg)	9.07	9.07	9.07	9.07	20.0	20.0
Starting pH	6.6	6.6	6.6	6.6	6.5	6.5
Starting titratable acidity	0.17	0.17	0.17	0.17	0.15	0.15
Ripening time (min)	60	65	60	65	30	30
pH at time of renneting	6.3	6.5	6.3	6.5	6.4	6.4
Titratable acidity	0.18	0.18	0.18	0.18	0.17	0.17
Renneting time (min)	40	35	40	35	35	35
pH at drain	6.3	6.4	6.3	6.3	5.8	5.9
Whey recovered (L)	ND[1]	ND	ND	ND	16.3	16.6
Curd recovered						
Before milling (kg)	0.94	1.20	1.18	1.46	2.64	2.10
After pressing (kg)	0.61	0.72	0.88	1.02	1.94	1.62
Yield (%)	6.7	7.9	9.7	11.2	9.7	8.1

[1]ND = not determined.

Table 4. Distribution of lysostaphin in different fractions collected during cheesemaking using milk from transgenic cows 312_T or 101_T[1]

Fraction	Concentration (SEM) of lysostaphin in fractions, µg of lysostaphin per mL of milk or per g of cheese			Recovery of lysostaphin based on initial amount in starting milk (%)		
	312–0% fat	312–3% fat	101–3% fat	312–0% fat	312–3% fat	101–3% fat
Pasteurized milk	0.87[c] (0.05)	1.11[bc,z] (0.08)	7.75[xy] (0.31)	100	100	100
Cheese milk	0.81[c] (0.09)	0.80[c,z] (0.11)	7.30[xy] (0.52)	93	72	93
Whey from vat	0.24[c] (0.09)	0.20[z] (0.12)	0.70[z] (0.05)	10	13	8
Whey, total				12	15	10
Curd from vat	7.58[ab] (1.02)	9.06[a,xy] (1.56)	26.9[w] (0.55)	90	87	36
Cheese 1	6.25[b] (0.31)	6.72[ab,yz] (0.47)	19.3[wx] (0.47)	48	55	21
Cheese 90	4.30[bc] (0.55)	4.38[bc,yz] (0.00)	16.7[wx] (0.86)	33	36	21

[a–c]Means in columns for cheese from 312_T, milk with either 0 or 3% fat, with different letters are different ($P < 0.05$); the lack of letters in a column indicates no differences were found.

[w–z]Means in columns for cheese made with milk containing 3% fat, either from 312_T or 101_T, with different letters are different ($P < 0.05$); the lack of letters in a column indicates no differences were found.

[1]Milk contained 0 or 3% fat and was pasteurized at 72°C for 15 s. Fractions collected included inoculated cheesemilk 30 min after adding starter cultures, whey drained from vat, total whey included whey from overnight pressing, curd after draining the whey and at pH near 5.2, and pressed cheese stored for 1 or 90 d at 4°C.

from cow 310_C and 312_T, the control cheeses had the greater yields, whereas between cow 101_T and 207_C, the cheese containing lysostaphin had the greater yield.

The concentration of the lysostaphin in different fractions varied as the milk was processed into cheese. Based on the initial concentration in the pasteurized milk, lysostaphin concentrations (Table 4) and activities (Figure 3, columns 5 and 6) were not significantly different ($P < 0.05$) after milks were incubated with starter cultures. Cheeses made side-by-side at the same time had similar increases in titratable acid and decreases in pH (Table 3). Lysostaphin activity also decreased following exposure to rennet (Figure 3, columns 6 and 7); see discussion in the next paragraph. The whey drained from the vat had low concentrations of lysostaphin (Table 4), but because of the volume, accounted for approximately 8 to 13% of the initial lysostaphin. The majority of the remaining lysostaphin was concentrated in the curd (Table 4). Milling and overnight pressing reduced the weight of the recovered curd by 25 to 40% and further reduced the amount of lysostaphin in the cheese curd. Compared with the whey removed from the vat, the small amount of whey removed from the curd during overnight pressing had greater activities (Figure 3, column 8 versus 10) and only accounted for an additional 2% recovery; 10 to 15% in total whey. The reduction of lysostaphin levels in the curd was dramatic. For cow 101_T, which produced the greater level of lysostaphin, only 36% of the initial amount of enzyme in the cheese vat was recovered in the curd and 10% in the total whey. It is unclear why the 101_T cheese lost such a large percentage of the lysostaphin during cheesemaking steps. For cow 312_T, which secreted the lesser level of lysostaphin, almost 90% of the enzyme was recovered in the curd with ap-

proximately 10% recovered in the whey. Metcalf and Deibel (1969) also found that the majority of bacterial lysostaphin activity was associated with the curd and that 7% of the enzyme activity was found in the whey. The samples with particulate matter (curd and cheese samples) were problematic when conducting the ELISA assays and could account for some of the variation in lysostaphin concentration.

Composition of the different fractions collected during heat processing and cheesemaking were similar among cows with expected shifts in composition because of processing (data not shown). The cheesemilks were uniform except for the fat content. All milks contained 3.6 to 3.8% protein and 4.6 to 4.8% lactose. The 0% fat cheesemilks contained 9.0% total solids and 0.1 to 0.2% milkfat. The standardized milks contained approximately 12% total solids with 2.9 to 3.0% milkfat. Whey fractions contained greater proportions of whey proteins and less caseins and fat, whereas curd fractions contained greater amounts of caseins and fat (in standardized fat samples).

The cheeses made from milk with the same level of fat were similar in composition (Table 5). Cheeses made from milk containing 3% fat had compositions typical of semi-hard cheeses: 42 to 44% moisture, 24 to 25% fat, and 25 to 27% protein. As expected, the nonfat cheeses had greater moisture and protein concentrations than the full fat cheeses ($P < 0.05$). There were slight batch-to-batch variation among the cheeses, but no consistent differences ($P < 0.05$) were noted between cheeses made with milk from control or transgenic cows.

Cheeses from cow 312_T contained 6.3 to 6.7 µg of lysostaphin/g of cheese, with little difference between the cheeses made from milk containing 0 or 3% fat. Despite the significant loss of lysostaphin during the
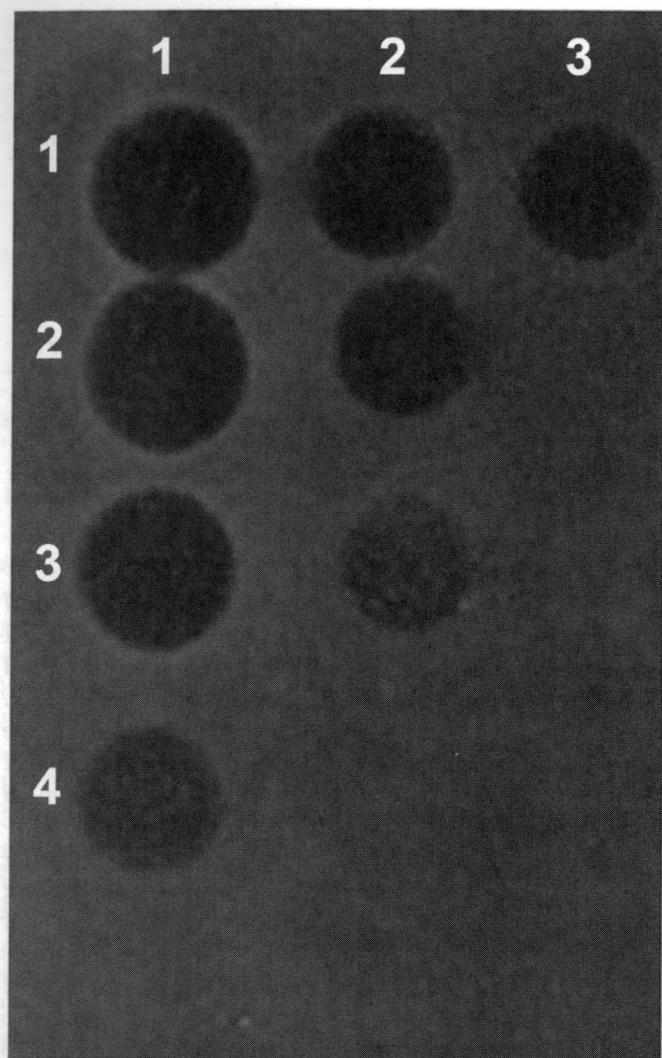
Figure 5. Spot-on-lawn assay showed the decrease in activity of bacterial lysostaphin after incubation with chymosin in 10 mM phosphate buffer, pH 6.0, 90 min at 37°C. Lysostaphin concentration decreased from left to right [1) 50, 2) 25, and 3) 12.5 µg of lysostaphin/mL], and chymosin concentration increased from top to bottom [a) 0, b) 7.8, c) 15.5, d) 31 µg of chymosin/mL].

manufacturing steps, cheese from 101_T contained 16.7 µg of lysostaphin/g of cheese. Based on the total amount of lysostaphin present in the cheese vat at the start of the process, pressed cheese curds from cow 321_T retained 48 and 55% of the enzyme (made from 0 and 3% fat milk, respectively) and cheese curds from 101_T retained 21% of the enzyme. The amount of lysostaphin decreased slightly after storage at 4°C (Table 4), but all cheeses still contained sufficient quantities of lysostaphin to inhibit the grow of *S. aureus* in both the SOL (Figure 3, columns 11 and 12) and MIC (Figure 4, rows 1 to 4) assays.

To determine if lysostaphin could alter cheesemaking properties, a series of tests was conducted. Bacterial lysostaphin in a phosphate buffer was incubated with chymosin at concentrations similar to that used in cheesemaking. The loss of lysostaphin activity was shown to be concentration-dependent (Figure 5). Lysostaphin is known to be inactivated by enzymes, [i.e., trypsin (Schindler and Schuhardt, 1965)] and contains several sites that could be susceptible to hydrolysis by chymosin [phe284 - phe285, phe285 - met286, and leu330 - ser331]. Therefore, exposure to chymosin in the cheese vat and cheese matrix during aging may have contributed to the reduction in lysostaphin content. In other studies of transgenic mice expressing human lysozyme in the milk, the presence of the lysozyme reduces the rennet (vegetable source) coagulation time and increases the milk gel strength as well as reduces the growth of cold spoilage bacteria (Maga et al., 1995, 1998, 2006).

To determine if the presence of lysostaphin in the milk would influence the ability of chymosin to coagulate milk, another set of control experiments was conducted. Pasteurized 0 and 3% fat milk from transgenic and control cows was treated with chymosin and gelation time compared. As found in the analysis of the composition, individual cows had main effects, but no differences were found between transgenic and nontransgenic milks ($P < 0.05$; Table 6). In the actual cheese trials, a slight decrease in lysostaphin activity was detected following

Table 5. Composition of the different cheeses made with cheese milk (3 or 0% fat) from nontransgenic control ($_C$) or transgenic ($_T$) cows[1]

Item	Fat in milk (%)	Moisture (%)	Fat (%)	Protein (%)	Lactose (%)	Lysostaphin (µg/g)
312_T	0	57. 5^a (0.7)	0.1^b (0.0)	36.2^a (1.5)	0.50 (0.08)	6.3 (0.3)
310_C	0	55.6^a (1.6)	0. 2^b (0.1)	38.8^a (1.6)	0.31 (0.05)	0
312_T	3	43.5^b (0.5)	24.9^a (1.0)	25.7^b (0.9)	0.64 (0.06)	6.7 (0.5)
310_C	3	43.0^b (1.2)	24.2^a (1.1)	26.6^b (0.7)	0.32 (0.08)	0
101_T	3	42.3 (0.7)	24.9 (0.9)	27.0 (0.7)	0.69 (0.13)	16.7 (0.5)
207_C	3	42.3 (0.6)	25.4 (0.2)	26.7 (0.8)	0.72 (0.05)	0

$^{a-d}$Means in the same column for cheese from 312_T and 310_C with different letters are different ($P < 0.05$); a lack of letters in the column for lactose indicates no differences were found.

[1]Cow 312_T was paired to 310_C, and cow 101_T was paired to 207_C. Numbers in parentheses are SEM.
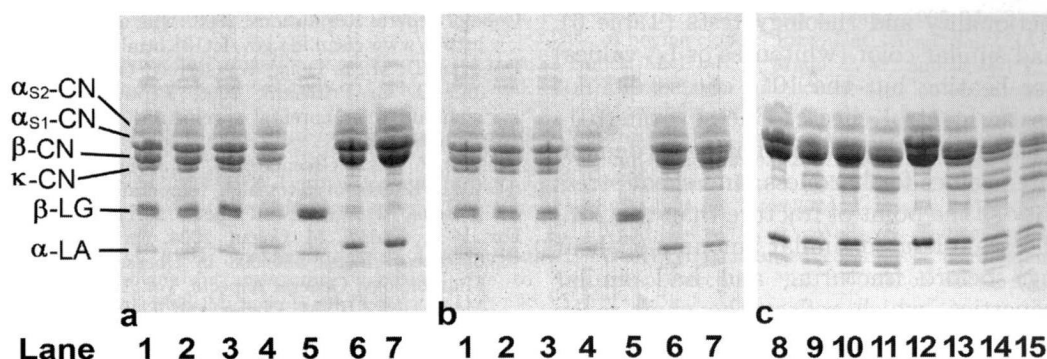
Figure 6. Protein profiles of different fractions from a) 3% fat milk from nontransgenic cow 310_T; b) 3% fat milk from transgenic cow 312_C; and c) cheeses made from 3% milk without (lanes 8 to 11) and with lysostaphin (lanes 12 to 15). Lanes: 1) raw milk, 2) HTST pasteurized milk, 3) cheesemilk incubated with starter cultures, 4) gelled milk gel after the addition of rennet, 5) whey, 6) fresh curd, and 7, 8, and 12) cheese after 1 d of manufacture, 9 and 13) cheese after 30d of aging, 10 and 14) cheese after 60 d of aging, and 11 and 15) cheese after 90 d of aging. Protein bands are identified as α_{s2}-CN, α_{s1}-CN, β-CN, κ-CN, β-LG, and α-LA.

the renneting of the cheese milk (Figure 3, columns 6 and 7). Chymosin (rennet, EC 3.4.23.4) is the primary enzyme used to coagulate milk and contributes to the hydrolysis of the cheese protein matrix during aging to obtained desired sensorial, functional, and textural attributes. The SDS-PAGE analysis of different fractions indicated protein distribution typical of the fractions with no differences noted between transgenic or control cows (Figure 6a and 6b). Protein profiles from the cheeses showed the proteolytic breakdown of the caseins expected in aging cheeses (Figure 6c). Cheeses made with milk from transgenic cows showed more peptide bands than the control cheeses, which indicated a higher degree of proteolysis than the paired control cheese. Therefore, lysostaphin did not appear to have reduced the chymosin degradation of the proteins in the cheese matrix.

Earlier studies have shown that lysostaphin acitivity decreases as the pH is lowered from 7.5 (Schindler and Schuhardt, 1965; Metcalf and Deibel, 1969). Milk has a pH of 6.6 and gradually decreases throughout the cheese making process; a final pH of 5.2 is typical for many semi-hard cheeses. Schindler and Schuhardt (1965) reported that decreasing pH in cheese was the main reason cheeses made from milk containing 5 µg of bacterial lysostaphin/mL and inoculated with over 5×10^4 *S. aureus* counts/mL would eventually support large *S. aureus* populations. They claimed that lysostaphin was effective only in the initial fermentation stages of cheesemaking. With improved detection methods and activity assays, our study showed a decrease in activity through the cheesemaking steps but also showed that recombinant lysostaphin was still present in cheeses with pH as low as 4.9 and would still inhibit *S. aureus* growth. This activity persisted in cheeses aged up to 90 d at 4°C.

Cheeses made from milk containing 3% fat from 101_T and 207_C were the only cheeses with enough material

Table 6. Functional and rheological properties of cheeses made from 3% milk from transgenic (101_T) and nontransgenic (207_C) cows

Item	101_T		207_C	
	Mean	SEM	Mean	SEM
Chymosin coagulation time[1] (min)	11.2	0.20	10.07	0.20
Meltability	1.33[b]	0.09	2.07[a]	0.09
Hardness (N)	73.1[a]	3.99	37.4[b]	1.1
Cohesiveness	0.22	0.01	0.24	0.01
Springiness (mm)	7.26	0.45	8.47	0.27
Elastic modulus (kPa)	14.1	1.80	22.4	3.20
Viscous modulus (kPa)	4.85	0.75	7.59	0.68
Shear stress (kPa)	38.3[a]	0.66	18.0[b]	0.39
Shear strain	0.83	0.04	0.71	0.02
Shear rigidity (kPa)	46.2[a]	1.88	25.4[b]	1.30

[a,b]Means in the same row with different letters are different ($P < 0.05$); a lack of letters in rows indicates no differences were found.

[1]Coagulation times collected on all milks used to make the 6 cheeses.

to conduct functionality and rheology tests (Table 6). Both cheeses had similar color (whiteness or L* values) before and after heating but the 101_T cheese did not melt as well as the control cheese. Cheese from 101_T had a significantly stronger protein matrix as demonstrated by greater values for hardness, and shear stress and shear rigidity at the point of fracture than the 207_C cheese. Both cheeses tolerated similar amounts of shear strain (twisting) before fracturing and had similar viscoelastic properties, which reflect the short-range internal interactions within the protein matrix. Both cheeses had similar cheesemaking details (ripening times with starter culture before addition of chymosin, same coagulation times, and similar pH and titratable acidity changes; Table 3) and compositions, but varied in yield and rheology that suggest subtle differences in the formation of the cheese matrix. Although this study was undertaken to determine the fate and distribution of lysostaphin in processed dairy foods, comparison of functional and rheological properties suggest that lysostaphin may also influence other properties within the cheese matrix.

CONCLUSIONS

This study showed that lysostaphin secreted into the milk of transgenic cows persisted through typical dairy processing procedures, although at reduced levels, and remained viable in pasteurized milk, cheese, and whey. The lysostaphin did not hinder the coagulation of milk or inactivated the starter cultures used in the cheesemaking process. Enough of the lysostaphin survived typical dairy processing conditions to inhibit *S. aureus* growth and impart potential value as a bioprotective agent against staphylococci in dairy foods. Further research is needed to evaluate its impact on food safety and quality.

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